

REMARKS

Entry of the foregoing, reexamination and further and favorable reconsideration of the subject application in light of the following amendment and remarks, pursuant to and consistent with 37 C.F.R. § 1.112, are respectfully requested.

As correctly indicated in the Official Action Summary, claims 1-26 were pending in this application when last examined. Of the pending claims, claims 1-10, 13-16, and 23-26 were withdrawn as being drawn to a non-elected invention and claims 11, 12 17-22 were examined on the merits.

By the foregoing amendment amends claims 11, 17, and 18 have been amended and new claims 27-37 have been added. The present amendment also cancels claims 21 and 22 without prejudice or disclaimer to the subject matter contained therein. Applicants reserve the right to file a continuation or divisional on any of the canceled subject matter.

Support for the amendment to claims 11 and 17 adding the phrase "isolated" can be found at least on page 14, lines 14-34, and page 15, lines 6-21. Support for the amendment to claims 11 and 17 adding the phrase ", wherein said lipid is obtainable by extracting microbial cells with an organic solvent" can be found at least on page 15, lines 6-21. Support for the definition of the microorganisms in claim 17 can be found at least in original claims 13-16. Support for the amendment to claim 18 can be found at least on page 4, lines 1-5. Support for the newly added claims can be found at least in original claims 1-5 and 13-16. Accordingly, no new matter has been added by the amendments to the claims. Moreover, the foregoing amendments to the claims were not intended to narrow the scope of any of the claims.

Rejections Under 35 U.S.C. § 101

Claims 11-12 and 17-22 have been rejected under 35 U.S.C. § 101 for allegedly reading on a product of nature. Official Action, page 3. This rejection is respectfully traversed. However, to expedite prosecution, and not to acquiesce to the Examiner's rejection, claims 11 and 17 have been amended to include the word "isolated" as suggested. Therefore, the rejection has been rendered moot and withdrawal of such rejection is respectfully requested.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 11, 12, and 17-22 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Official Action, pages 3 and 4. Applicants respectfully traverse this rejection.

In order to expedite prosecution, and not to acquiesce to the Examiner's rejections, claims 21 and 22 have been canceled without prejudice or disclaimer. Accordingly, the Examiner's allegation that claims 11 and 12 are substantial duplicates of claims 21 and 22 has been rendered moot.

The Examiner has asserted that claims 11, 12, and 17-22 are "confusing in that it is not specified whether the weight percent is dry or wet weight." It is well established that claim language should be read in light of the specification as it would be interpreted by one of ordinary skill in the art. See, e.g., In re Sneed, 218 U.S.P.Q. 385, 388 (Fed. Cir. 1983). One of ordinary skill in the art would certainly understand what was meant by the

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language of claims 11, 12, and 17-22. Thus, contrary to the Examiner's allegations, such claims would not be confusing to one of skill in the art in view of the specification.

As to the Examiner's concern about claims 17 and 18 depending upon a non-elected claim, Applicants note that the claims have been amended to remove this dependency. Accordingly, this rejection has been rendered moot.

Rejections Under 35 U.S.C. § 102 or 35 U.S.C. § 103

Claims 11-12 and 17-22 have been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by, or in the alternative, under 35 U.S.C. § 103(a) as allegedly obvious over Totani et al., IND. APPL. SINGLE CELL OILS, Ed. Kyle et al., American Oil Chemists Society, Champaign, Illinois, pp. 52-60 (1992) ("Totani"). Official Action, pages 3 and 4. This rejection is respectfully traversed.

Claims 11-12 and 17-22 have also been rejected under 35 U.S.C. § 102(b) as being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as being obvious over Li et al., THE CANADIAN JOURNAL OF CHEMICAL ENGINEERING, 73:135-139 (1995) ("Li"). Official Action, pages 5 and 6. This rejection is also respectfully traversed.

According to the Examiner, Totani at page 59, second paragraph, allegedly discloses a microbial lipid containing arachidonic acid which appears to be identical to the presently claimed microbial lipid containing arachidonic acid because the referenced lipid isolated from *Mortierella* contains 90% arachidonic acid. Similarly, the Examiner alleges that at page 138, first full paragraph and Table 6, Li discloses a microbial lipid containing arachidonic acid which appears to be identical to the presently claimed microbial lipid

containing arachidonic acid because the referenced lipid isolated from *Mortierella* contains 72.5% arachidonic acid. Thus, the Examiner believes that the cited references anticipate the claimed microbial lipid containing arachidonic acid because the references purportedly disclose a microbial lipid containing arachidonic acid which is of the same class and origin as the claimed microbial lipid and which appears to be identical to the claimed microbial lipid containing arachidonic acid. The Examiner further believes that even if the claimed microbial lipid containing arachidonic acid is not identical to the referenced ones, the differences are allegedly so slight that the referenced microbial lipids are likely to inherently possess the same properties of the claimed microbial lipid. As mentioned above, Applicants respectfully traverse both of these rejections.

As is evident from the discussion above, the Examiner has applied the same reasoning for both prior art rejections. Accordingly, Applicants address both rejections together below.

To anticipate a claim, a single prior art reference must teach, either expressly or inherently, each and every element of the claimed invention. See M.P.E.P. § 2131; Verdegaal Bros. v. Union Oil Co. of California, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987); Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1379, 231 U.S.P.Q. 81, 90 (Fed. Cir. 1986).

In addition, to establish a *prima facie* case of obviousness, three criteria must be met. First, there must be some suggestion or motivation in the reference to combine either the references themselves or to modify or combine the reference teachings. See M.P.E.P. § 2143; In re Vaeck, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991). This element

requires that the Examiner must show "some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art that would lead that individual to combine the relevant teachings of the references." In re Fine, 837 F.2d 1071, 1074, 5 U.S.P.Q.2d 1596, 1598 (Fed. Cir. 1988). In other words, the Examiner must provide a logical reason as disclosed in the prior art at the time of the invention for combining the references along the lines of the invention; otherwise the use of such teachings as evidence of non-obviousness will entail impermissible hindsight. Ex parte Stauber and Eberle, 208 U.S.P.Q. 945, 946 (Bd. App. 1980). Second, the prior art must provide a reasonable expectation of success. See M.P.E.P. § 2143.02; Vaeck, 947 F.2d at 488, 20 U.S.P.Q.2d at 1438; In re Merck & Co., Inc., 800 F.2d 1091, 231 U.S.P.Q. 375 (Fed. Cir. 1986). Third, the prior art references must teach or suggest each and every element of the claimed invention. See M.P.E.P. § 2143.03; In re Royka, 490 F.2d 981, 180 U.S.P.Q. 580 (C.C.P.A. 1974); In re Wilson, 424 F.2d 1382, 1385, 165 U.S.P.Q. 494, 496 (C.C.P.A. 1970).

For the reasons noted below, the cited references fail to either anticipate or render obvious the claimed invention because they fail to teach or suggest each and every limitation of the claimed invention.

Applicants note that the references cited by the Examiner describe a method for the purification of fatty acids by hydrolyzing a lipid and then concentrating the hydrolyzed product by urea treatment. As a result, the concentrated product of the cited art references is a mixture of free fatty acids.

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On the other hand, as can be seen from the enclosed reference Shimizu et al., J. AM. OIL CHEM. SOC., 68(4):254-158 (1991) ("Shimizu") at page 256, second column, lines 9 to 10, and at page 257, Table 5, lipid obtained from microorganisms, such as *Mortierella* comprises triglycerides as the main component and phospholipids as a minor component, but does not contain free fatty acids. Therefore, the lipid of claims 11, 12, 17-20 and 30-33 is different from the concentrated product (i.e., a mixture of fatty acids) of both Totani and Li.

Moreover, the lipid of the present invention which contains a high ratio of arachidonic acid is obtained by culturing a mutant strain wherein $\omega 3$ desaturase activity has been decreased under the specific temperature condition of the claimed invention (new claims 27-39) and as described on page 12, lines 3 to 12, Examples 2 to 6. The cited references on the other hand do not describe nor suggest the novel means used in the present invention. Accordingly, the methods described in the prior art references do not produce the claimed lipid of the present invention. In addition, the cited references fail to describe or suggest the lipid of claims 27-29 and 34-39.

CONCLUSION

From the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order, and such action is earnestly solicited.

In the event that there are any questions relating to this Amendment and Reply, or the application in general, the Examiner is invited to telephone the undersigned concerning such questions so that prosecution of this application may be expedited.

Respectfully submitted,

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Attachment to Amendment and Reply dated May 29, 2002

Marked-up Copy of Amended Claims 11, 17, and 18

([bracketed] items deleted; underlined items added)

11. (Amended) An isolated arachidonic acid-containing microbial lipid containing 72% by weight or more of arachidonic acid to the total fatty acids in said lipid, wherein said lipid is obtainable by extracting microbial cells with an organic solvent".

17. (Amended) [A] An isolated lipid containing arachidonic acid wherein the arachidonic acid content in the total fatty acids in the lipid is 50% by weight or more, said lipid being obtained by culturing a microorganism [according to any one of claims 13 to 16] in which ω 3 desaturase activity has been decreased or is lacking, wherein said lipid is obtainable by extracting microbial cells with an organic solvent, and further wherein said microrganism is obtained by the mutagenesis of a microrganism capable of producing arachidonic acid and belonging to the genus *Mortierella*, the genus *Conidiobolus*, the genus *Pythium*, the genus *Phytophthora*, the genus *Penicillium*, the genus *Cladosporium*, the genus *Mucor*, the genus *Fusarium*, the genus *Aspergillus*, the genus *Rhodotorula*, the genus *Entomophthora*, the genus *Echinosporangium* or the genus *Saprolegnia*.

Attachment to Amendment and Reply dated May 29, 2002

Marked-up Copy of Amended Claims 11, 17, and 18

([bracketed] items deleted; underlined items added)

18. (Amended) [A] The lipid containing arachidonic acid according to claim 17 [in which the arachidonic acid content in the total fatty acids in the lipid is 50% by weight or more and], wherein the percentage of eicosapentaenoic acid to the total fatty acids in the lipid is [content] is 0.5% by weight or less [, said lipid being obtained by culturing a microorganism according to any one of claims 13 to 16 in which ω3 desaturase activity has been decreased or is lacking].

Production of Odd Chain Polyunsaturated Fatty Acids by *Mortierella* Fungi

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A soil isolate, *Mortierella alpina* 1S-4, was found to show high production of odd chain polyunsaturated fatty acids (PUFAs) among various arachidonic acid-producing *Mortierella* strains tested. The fungus mainly accumulated 5,8,11,14-cis-nonenadecatetraenoic acid. With 5% n-heptadecane and 1% yeast extract as growth substrates, the amount of C_{19:4} acid accumulated reached 44.4 mg/g dry mycelia (0.68 mg/mL of culture broth). This value accounted for 11.2% of the total fatty acids in the extracted lipids from mycelia, and odd chain fatty acids comprised over 95% of the total mycelial fatty acids. The addition of sesamin, a specific inhibitor of Δ5 desaturation, caused an increase in C_{19:3} acid and an accompanying decrease in C_{19:4} acid. On the other hand, species of *Mortierella* that could not produce C-20 PUFAs accumulated C-17 acids, but no C-19 PUFAs, when grown with fatty substrates with an odd chain skeleton. The odd chain PUFAs were distributed in both neutral and polar lipids. The biosynthetic route to C_{19:4} acid was presumed to mimic the n-6 route to arachidonic acid as follows: C_{17:0} → C_{17:1} → C_{17:2} → C_{19:3} → C_{19:4} acids.

KEY WORDS: Arachidonic acid, biosynthesis of polyunsaturated fatty acids, *Mortierella alpina*, nonadecatetraenoic acid, odd chain polyunsaturated fatty acids.

The natural occurrence of straight-chain, odd-numbered polyunsaturated fatty acids (PUFAs) has been reported in ruminant, fish and several other animal lipids. They can be considered as normal constituents of such fats. However, the odd chain PUFAs reported accounted for only a small percentage of the total cellular fatty acids. Only mullet oil so far has been reported to be a relatively rich source of these fatty acids, such as C-17 and C-19 PUFAs (1). The fatty acid methyl esters containing mainly 9,12-C_{17:2} and 6,9,12-C_{17:3} acids prepared from mullet oil, as well as linoleic acid methyl ester, were shown to cure the external symptoms of fat-deficient rats and to be converted to 5,8,11,14-C_{18:4} acid in the rat liver (2,3). On the other hand, some mucoralean fungi (4) and *Candida* yeasts (5), grown with odd chain n-alkanes, have been shown to accumulate C-17 unsaturated fatty acids such as C_{17:1}, C_{17:2} and C_{17:3} acids. However, they do not produce C-19 PUFAs. In our recent studies (6-17), it was found that several fungal strains belonging to the genus *Mortierella* accumulate C-20 PUFAs, such as dihomoy-linolenic, arachidonic and eicosapentaenoic acids, in their mycelia. Here we report that these arachidonic acid producers accumulate specifically large amounts of C-17 and C-19 PUFAs in their mycelia when grown with odd chain

n-alkanes. The identification of the odd chain PUFAs and their distribution in fractionated lipids are also described.

MATERIALS AND METHODS

Chemicals. n-Alkanes were purchased from Wako Pure Chemicals (Osaka, Japan). (+)-Sesamin was prepared from unroasted sesame oil (S. Shimizu, K. Akimoto, Y. Shinmen, M. Sugano and H. Yamada, unpublished data). All other reagents used in this work were of analytical grade and commercially available.

Microorganisms. All fungal strains used were from our stock cultures (AKU Culture Collection, Faculty of Agriculture, Kyoto University). Each fungus was cultivated in a medium (10 mL, pH 6.0) containing glucose and/or odd chain fatty substrates, as the major carbon source, and 1% yeast extract in a 50-mL flask for 6-8 days at 28°C with reciprocal shaking (120 strokes/min).

Analysis of fatty acid composition and n-pentadecane. Extraction and determination of the mycelial fatty acids were described previously (11,12,14). n-Pentadecane was analyzed by gas liquid chromatography (GLC) in the same manner as for fatty acid methyl ester analysis except for the column temperature (140°C).

Lipid extraction and separation. Mycelia washed with water and n-hexane were treated twice with chloroform/methanol/water according to the procedure of Bligh and Dyer (18). The lipid extract was separated by thin-layer chromatography (TLC) on silica gel (60F₂₅₄, 200 × 200 × 0.25mm; E. Merck, Darmstadt, Germany). The spots were located under u.v. after spraying the plate with 0.2% dichlorofluorescein in ethanol. The solvent system used was petroleum ether/diethyl ether/acetic acid (82:18:1, v/v/v). The spots were directly scraped off for methanolysis.

Isolation of C_{19:4} acid methyl ester from fungal mycelia and other methods. The procedures used for transmethylation and purification of C_{19:4} acid were essentially the same as described previously (6). Measurements of mass and ¹H NMR (nuclear magnetic resonance) spectra, and other analyses were also carried out as described previously (6,11-14).

RESULTS

Formation of odd chain PUFAs from glucose. The mycelia of *Mortierella alpina* CBS 219.35 grown in a medium containing only glucose and yeast extract contained significant amounts of unusual fatty acids besides palmitic, stearic, oleic, linoleic, γ-linolenic, dihomoy-linolenic and arachidonic acids, which are commonly found in arachidonic acid-producing species of *Mortierella*. Two major components of these unusual fatty acids showed the same retention times as authentic C_{15:0} and C_{17:0} acid methyl esters, respectively, on both GLC and high performance liquid chromatography (HPLC). The others were also considered to be odd chain unsaturated fatty acids because of their retention times on GLC and HPLC, and the

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ODD CHAIN POLYUNSATURATED FATTY ACIDS

TABLE 1

Accumulation of Odd Chain PUFAs by Several Species of *Mortierella* in a Medium Containing Methyl Pentadecanoate or *n*-Pentadecane^a

Strain	FAM ^b or NA ^b added (%)	Mycelial fatty acid composition ^b (%)												Total odd chain FA	
		15:0	16:0	17:0	17:1	18:0	18:1	18:2	18:3	19:3	19:4	20:3	20:4		
Subgenus Mortierella															
<i>M. alpina</i> 1S-4 AKU 2998	FAM 8.6	13.8	4.7	11.7	6.8	2.3	9.6	4.7	2.8	1.9	7.4	2.2	22.9	9.3	50.7
	NA 2.0	24.8	1.0	14.0	15.0	tr	5.0	3.1	2.8	1.8	11.6	tr	10.2	10.7	74.5
<i>M. elongata</i> 1S-5 AKU 8999	NA 2.0	47.4	1.7	9.5	12.3	tr	6.8	1.5	1.6	1.6	5.4	0.7	7.2	4.3	78.6
<i>M. verticillata</i> IFO 8575	NA 2.0	35.2	1.9	8.8	13.6	tr	7.2	5.3	2.9	1.0	3.7	tr	15.3	5.6	68.1
<i>M. kuhlmanii</i> CBS 157.71	NA 2.0	47.2	2.9	7.2	7.9	1.2	5.2	4.0	2.1	1.3	3.4	tr	8.2	8.4	75.6
<i>M. alpina</i> CBS 219.35	FAM 1.0	51.6	8.3	6.2	5.7	1.2	9.5	6.2	3.7	0.9	1.1	1.6	7.3	1.7	67.0
Subgenus Micromucor															49.2
<i>M. ramaniana</i>	FAM 1.0	31.8	8.8	9.1	8.3	2.8	20.6	7.4	9.5	nd	nd	nd	nd	nd	0.9
var. <i>engulispora</i> IFO 8187	NA 2.0	2.6	13.7	nd	nd	1.9	40.7	17.4	22.8	nd	nd	nd	nd	nd	2.6
<i>M. isabellina</i> IFO 6738	FAM 1.0	28.1	10.1	2.5	2.7	1.8	29.1	12.7	11.7	nd	nd	nd	nd	nd	33.3

^a Each strain of *Mortierella* was grown in a medium containing 2% glucose, 1% yeast extract and methyl pentadecanoate or *n*-pentadecane, as indicated, pH 6.0, for 6-8 days at 28°C.

^b Abbreviations used: FAM, methyl pentadecanoate; NA, *n*-pentadecane; 15:0, pentadecanoic acid; 16:0, palmitic acid; 17:0, heptadecanoic acid; 17:1, heptadecenoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ -linolenic acid; 19:3, nonadecatrienoic acid; 19:4, nonadecatetraenoic acid; 20:3, dihomoy- γ -linolenic acid; 20:4, arachidonic acid; FA, fatty acids; tr, trace; nd, not detectable.

TABLE 2

Comparison of Mycelial Fatty Acid Composition of *M. alpina* 1S-4 Grown With Odd Chain Fatty Compounds^a

FAM or NA added ^b	Mycelial fatty acid composition ^b (%)												Total odd chain FA
	16:0	17:0	17:1	17:2	17:3	18:0	19:1	19:3	19:4	16:0	18:1	20:4	Others
C16 FAM	13.8	11.7	6.8	tr	1.2	6.2	1.9	7.4	4.7	9.6	22.9	19.8	60.7
C19 FAM	tr	tr	tr	nd	20.9	nd	nd	nd	13.2	8.6	42.2	15.1	20.9
C11 NA	0.5	0.7	0.2	nd	0.2	nd	nd	nd	17.5	25.0	28.3	27.6	1.7
C18 NA	1.1	1.2	0.3	nd	0.2	nd	nd	nd	16.7	18.7	33.9	29.9	2.8
C15 NA	16.8	8.4	6.6	tr	0.8	tr	1.2	3.3	8.2	11.4	25.2	19.1	36.6
C17 NA	2.0	9.4	4.6	1.2	1.0	tr	0.7	0.8	16.6	28.6	18.5	21.6	19.7
C19 NA	0.8	2.8	1.2	tr	2.4	tr	0.8	0.4	16.0	26.4	20.1	29.6	9.6

^a *M. alpina* 1S-4 was grown in a medium containing 2% glucose, 1% yeast extract and each of the indicated fatty acid methyl esters (0.5%) or *n*-alkanes (1.0%). pH 6.0, for 6-8 days at 28°C.

^b Abbreviations used: C16 FAM, methyl pentadecanoate; C19 FAM, methyl nonadecanoate; C11 NA, *n*-undecane; C13 NA, *n*-tridecane; C15 NA, *n*-pentadecane; C17 NA, *n*-heptadecane; C19 NA, *n*-nonadecane; 17:2, heptadecadienoic acid; 17:3, heptadecatrienoic acid; 18:0, nonadecanoic acid; 19:1, nonadecenoic acid. For other abbreviations, see Table 1.

results described later regarding their GLC-mass and ¹H NMR spectra. These odd chain fatty acids accounted for 17.7% of the total mycelial fatty acids, and comprised C_{15:0} (4.0%, by weight), C_{17:0} (7.2%), C_{17:1} (5.2%), C_{19:3} (0.8%) and C_{19:4} (0.5%) acids, whereas the odd chain fatty acids in mullet oil have been reported to account for about 25% of the total fatty acids, and to be comprised of C_{16:0} (11.2%), C_{17:0} (4.6%), C_{17:2} (2.6%), C_{19:4} (1.7%) acids, plus others (1). Similarly, *M. alpina* var. *renispora* CBS 210.32, *M. hyalina* NRRL 6427 and *M. minutissima* IFO 8573 accumulated detectable amounts of C-17 and C-19 PUFAs.

Conversion of fatty substrates with an odd chain skeleton to odd chain PUFAs. To obtain greater amounts of odd chain PUFAs, several species of *Mortierella* were grown with *n*-pentadecane or methyl pentadecanoate, as shown in Table 1. All the arachidonic acid-producing species of *Mortierella*, which belong to the subgenus *Mortierella*, accumulated C_{19:4} and C_{19:3} acids together with

C-16 and C-17 fatty acids, such as C_{16:0}, C_{17:0} and C_{17:1} acids. C-15 unsaturated fatty acids were not detected. On the other hand, species of *Mortierella* belonging to the subgenus *Micromucor*, which cannot produce C-20 PUFAs (10), accumulated C-17 PUFAs but not C-19 PUFAs. The total accumulation of odd chain fatty acids in arachidonic acid-producing species of *Mortierella* accounted for more than half of the total mycelial fatty acids. Both *n*-pentadecane and methyl pentadecanoate were efficiently converted to odd chain fatty acids, and exhibited essentially the same mycelial fatty acid profile. We selected *M. alpina* 1S-4 for the following experiments because of its high mycelial accumulation of C_{19:4} acid.

M. alpina 1S-4 was grown with glucose and odd chain fatty acid methyl esters or odd chain *n*-alkanes, as shown in Table 2. C-15 and C-17 alkanes were efficiently converted to C-19 PUFAs. But fatty substrates with shorter and longer carbon chains (i.e., C-11, C-13 and C-19) were

TABLE 3

Production of Nonadecatetraenoic and Arachidonic Acids by *M. alpina* 1S-4 Grown with *n*-Pentadecane or *n*-Heptadecane^a

(%)	<i>n</i> -Pentadecane				<i>n</i> -Heptadecane					
	Mycelial mass (mg/mL)	Mycelial PUFA content (mg/g dry mycelia)		PUFA yield (mg/mL)		Mycelial mass (mg/mL)	Mycelial PUFA content (mg/g dry mycelia)		PUFA yield (mg/mL)	
		19:4 ^b	Ara ^c	19:4	Ara		19:4	Ara	19:4	Ara
3	14.8	31.5	8.7	0.47	0.13	14.3	27.4	6.9	0.39	0.08
4	18.0	24.1	5.8	0.31	0.07	14.7	27.8	5.0	0.55	0.07
5	18.0	19.8	4.4	0.26	0.06	15.4	44.4	6.9	0.68	0.09

^a *M. alpina* 1S-4 was grown in a medium containing 3-5% *n*-pentadecane or *n*-heptadecane, as indicated, and 1% yeast extract, pH 6.0, for 7 days at 28°C.

^b 19:4, 5,8,11,14-cis-Nonadecatetraenoic acid.

^c Ara, arachidonic acid.

TABLE 4

Comparison of Mycelial Fatty Acid Composition of *M. alpina* 1S-4 Grown in the Presence or Absence of Sesamin^a

Addition of sesamin	Mycelial fatty acid composition ^b (%)													
	15:0	16:0	17:0	17:1	18:0	17:2	18:1	18:2	18:3	19:3	19:4	20:3	20:4	Others
No	15.1	9.1	8.9	5.1	5.3	1.6	9.6	4.5	3.8	1.0	3.4	3.8	26.3	3.0
Yes	20.0	9.8	7.2	3.1	2.8	1.1	6.7	1.7	4.4	3.8	1.7	14.1	19.6	4.0
Addition of sesamin	19:3/19:4	20:3/20:4	Odd chain fatty acids (%)			Even chain fatty acids (%)			S+M ^b (%)		P ^b (%)			
No	0.29	0.14	36.0			61.9			53.1		43.9			
Yes	2.24	0.72	37.3			59.1			49.6		46.4			

^a *M. alpina* 1S-4 was grown in a medium containing 2% glucose, 2% *n*-pentadecane and 1% yeast extract supplemented with 0.01% of sesamin or unsupplemented, pH 6.0, for 6 days at 28°C.

^b Abbreviations used: S, saturated fatty acids; M, monounsaturated fatty acids; P, fatty acids having more than two double bonds. For other abbreviations, see Tables 1 and 2.

not efficiently converted. When it was grown with C-15 or C-17 fatty substrates, the predominant odd chain fatty acids were C_{18:0}, C_{17:0}, C_{17:1}, C_{17:2}, C_{17:3}, C_{18:2} and C_{19:4} acids. C-15 unsaturated fatty acids and odd chain fatty acids of more than 21 carbons were not detected.

The data in Table 3 show that *M. alpina* 1S-4 could grow in a medium containing an odd chain *n*-alkane as a major carbon source and that the C_{18:4} acid content reached 44.4 mg/g dry mycelia (0.68 mg/mL of culture broth) when it was grown in a medium containing 5% *n*-heptadecane and 1% yeast extract. The total odd chain fatty acids accumulated reached over 95% of the mycelial fatty acids. The C_{18:4} acid accounted for 11.2%, whereas arachidonic acid accounted for only 1.5%.

Inhibitory effect of sesamin on desaturation of C_{15:3} acid to C_{18:4} acid. *M. alpina* 1S-4 was grown with *n*-pentadecane supplemented with 0.01% of (+)-sesamin or unsupplemented, as shown in Table 4. (+)-Sesamin and related lignan compounds are specific inhibitors of Δ5 desaturation (S. Shimizu et al., unpublished data). Table 4 shows that the mycelial "19:3/19:4" ratio increased from 0.29 to 2.24 with the supplementation of sesamin. The

mycelial "20:3/20:4" ratio increased from 0.14 to 0.72, without a change in the percentage of odd chain fatty acids (36% with no supplementation and 37% with supplementation) or that of PUFA (44% with no supplementation and 46% with supplementation).

Distribution of odd chain PUFA in extracted lipids. The lipids extracted from mycelia of *M. alpina* 1S-4 grown with 3% *n*-pentadecane (NA medium) or 2% glucose (G medium) were separated into the triacylglyceride (TG)-fraction (Rf=0.6), the polar lipid (PL)-fraction (Rf=0.02) and the *n*-alkane (NA)-fraction (Rf=0.85) (Table 5). The *n*-pentadecane remaining in the NA medium amounted to 6.1% of the supplemented *n*-pentadecane and mycelial *n*-pentadecane represented by the NA-fraction to 12.6%, so about 80% of the supplemented *n*-pentadecane was consumed during the cultivation and 12% of the consumed *n*-pentadecane was converted to mycelial fatty acids.

In the case of the NA medium, every odd chain fatty acid was found in both the TG- and PL-fractions, but the fatty acid compositions of these two fractions were different. Table 5 shows that the percentage of saturated

ODD CHAIN POLYUNSATURATED FATTY ACIDS

TABLE 5

Comparison of Mycelial Fatty Acid Composition of the TG^a-Fraction and PL^a-Fraction of *M. alpina* 1S-4 Grown in the Presence or Absence of *n*-Pentadecane^b

Medium ^c	Fra ^d	FA (mg/g dry mycelia)	Fatty acid composition ^d (%)											
			16:0	16:0	17:0	17:1	18:1	17:3	18:2	19:2	19:4	20:3	20:4	Others
NA	TG	218.7	56.8	1.2	21.0	7.7	1.9	2.3	0.4	1.0	1.7	tr	0.4	5.6
NA	PL	28.9	15.4	2.4	7.5	19.7	12.6	8.4	8.1	2.1	14.0	0.5	2.9	9.9
G	TG	173.6	nd	18.2	nd	nd	17.5	nd	5.6	nd	nd	2.3	38.7	17.8
G	PL	27.5	nd	16.0	nd	nd	16.8	nd	5.7	nd	nd	2.5	42.4	17.6

^aAbbreviations used: TG, triacylglyceride; PL, polar lipid.

^b*M. alpina* 1S-4 was grown in the NA or G medium for 6 days at 28°C.

^cThe NA medium contained 2% *n*-pentadecane and 1% yeast extract, pH 6.0. The G medium contained 2% glucose and 1% yeast extract, pH 6.0.

^dFra, fraction. For other abbreviations used, see Tables 1 and 2.

odd chain fatty acids in the TG-fraction was high (C_{15:0}, 56.8%; and C_{17:0}, 21.0%) and the percentage of PUFA_s was low (C_{19:4}, 1.7%; and C_{20:4}, 0.4%). On the other hand, the percentage of saturated odd chain fatty acids in the PL-fraction was relatively lower (C_{15:0}, 15.4%; and C_{17:0}, 7.5%) and that of PUFA_s was higher (C_{19:4}, 14.0%; and C_{20:4}, 3.9%). In the case of the G medium, the fatty acid compositions of both fractions were almost the same, the C_{20:4} acid content reaching about 40%. The ratio of the TG- and PL-fractions of the lipids from mycelia grown in the NA medium was 88:12, while that of the lipids from mycelia grown in the G medium was 36:14.

Identification of C_{19:4} acid and the other odd chain PUFA_s. C_{19:4} acid methyl ester (13 mg) was isolated from the lipids extracted from 40 g of wet mycelia of *M. alpina* 1S-4 grown in a medium containing 4% *n*-pentadecane and 1% yeast extract, pH 6.0, for 8 days at 28°C as described previously (6). The mass spectrum of the isolated methyl ester showed peaks at m/z 304 (M⁺; relative intensity, 2%), 260, 220, 180, 163, 150, 136, 119, 106, 93, 79 (base peak), 67, 55 and 41. The ¹H NMR spectrum in CDCl₃, with tetramethylsilane as an internal standard, showed signals at 0.90 (t, 3H, CH₃), 1.81 (m, 4H, CH₂), 1.68 (m, 2H, CH₂), 2.09 (m, 4H, CH₂), 2.30 (t, 2H, CH₂), 2.83 (m, 6H, CH₂), 3.63 (s, SH, CH₃) and 5.38 ppm (m, 8H, C=C). which are the same as those in the case of authentic methyl arachidonate except that the peak at 1.31 ppm of methyl arachidonate, corresponding to methylene protons at the 17, 18 and 19 carbon atoms, indicates 6H. These data suggest that the structure of the isolated methyl ester was 5,8,11,14-cis-nonadecatetraenoic acid methyl ester. The other odd chain PUFA methyl esters were partly purified and determined by GLC-mass spectra to be as follows: nonadecatrienoic acid methyl ester, m/z 306 (M⁺; relative intensity, 17%), 275, 222, 177, 163, 149, 136, 121, 107, 93, 79 (base peak), 67, 55 and 41; heptadecatrienoic acid methyl ester, m/z 278 (M⁺, 13%), 194, 163, 149, 136, 120, 107, 98, 79, 67 (base peak), 55 and 41; heptadecadienoic acid methyl ester, m/z 280 (M⁺, 22%), 249, 206, 164, 150, 136, 123, 109, 95, 81, 67 (base peak), 55 and 41.

DISCUSSION

Hoffmann and Rehm reported that three fungi belonging to the Mucorales, *Absidia spinosa*, *Cunninghamella*

echinulata and *Mortierella isabellina*, which could not produce C-20 PUFA_s, accumulated C-17 PUFA_s but no C-19 PUFA_s when grown with odd chain n-alkanes (4). Here we also show that *M. isabellina* and *M. ramaniana* var. *angulispora*, belonging to the subgenus *Micromucor*, which could not produce C-20 PUFA_s, could accumulate some C-17 acids but no C-19 PUFA_s. Some *Candida* yeasts, which are unable to form C-20 PUFA_s, were also reported to produce C-17 acids but no C-19 PUFA_s when grown on odd chain n-alkanes (5). These data indicate that only microorganisms that can produce C-20 PUFA_s can produce C-19 PUFA_s, and that microorganisms that are unable to produce C-20 PUFA_s cannot produce C-19 PUFA_s.

One of the predominant C-19 PUFA_s produced by arachidonic acid-producing species of *Mortierella* was identified as 5,8,11,14-cis-nonadecatetraenoic acid, corresponding to arachidonic acid minus one carbon unit at the ω-terminal. The other odd chain PUFA_s formed by these fungi were considered to be C_{19:3}, C_{17:3} and C_{17:2} acids, according to the mass spectra of their methyl esters. C-21 PUFA_s have not been detected. These data suggest that the odd chain PUFA_s were biosynthesized through the proposed pathway in Figure 1, which mimics the pathway for the biosynthesis of even chain PUFA_s, the following successive reactions being involved: Oxidation of *n*-pentadecane to C_{18:0} acid, elongation to C_{17:0} acid, desaturation to 6,9,12-cis-C_{17:3} acid via C_{11:1} and C_{11:2} acids as successive intermediates, elongation of C_{17:3} acid to C_{19:3} acid, and further desaturation of C_{19:3} acid to C_{19:4} acid. These odd chain PUFA_s correspond to the equivalent even chain PUFA_s minus one carbon unit at the ω-terminal. This is supported by the fact that only microorganisms that can produce C-20 PUFA_s can produce C-19 PUFA_s. The reports (2,3) that 9,12-C_{17:3} and 6,9,12-C_{17:3} acids were probably converted to 5,8,11,14-C_{19:4} acid in the rat liver, and the results in Table 3 that show a significant decrease in the accumulation of arachidonic acid in *M. alpina* 1S-4 when a large amount of C_{19:4} acid is produced also support this assumption.

We reported that (+)-sesamin and some related lignans are specific inhibitors of the Δ5 desaturation reaction in *M. alpina* 1S-4 and rat liver (S. Shimizu, et al., unpublished data). Mycelial dihomoo-γ-linolenic acid, which is a substrate for Δ5 desaturation, increases with an accompanying decrease in mycelial arachidonic acid, a product

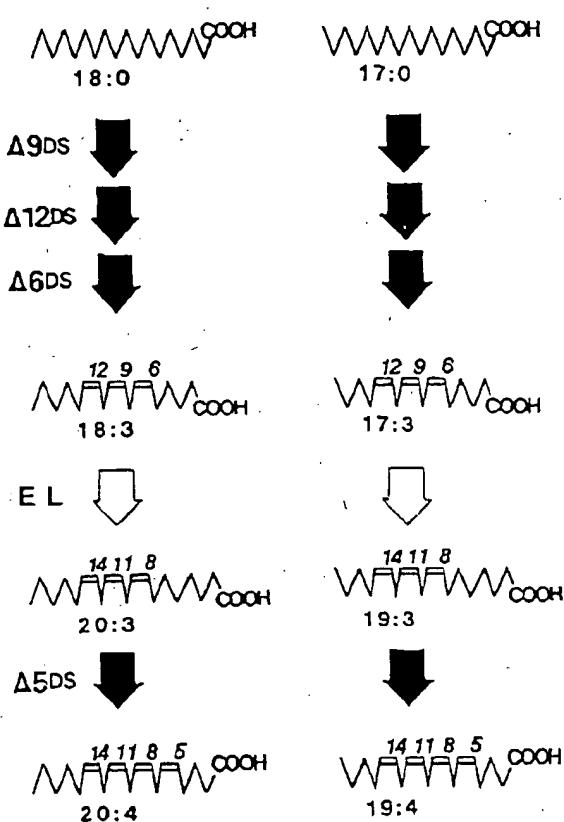


FIG. 1. Proposed pathway for the biosynthesis of odd chain PUFAs in *Mortierella*. Abbreviations used: DS, desaturation; EL, elongation. For other abbreviations, see Tables 1 and 2.

of Δ5 desaturation. The composition of the other mycelial fatty acids does not change when the fungus is grown with (+)-sesamin. Here we show that mycelial C_{19:3} acid also increased with an accompanying decrease in C_{19:4} acid when *M. alpina* 1S-4 was grown with *n*-pentadecane in the presence of (+)-sesamin. This phenomenon suggests that both the desaturation of C_{18:3} acid to C_{18:4} acid and that of dihomo-γ-linolenic acid to arachidonic acid are catalyzed by the same enzyme. This also supports the above assumption that the biosynthesis of odd chain PUFAs mimics that of even chain PUFAs.

About 90% of the supplemented *n*-pentadecane was incorporated into the mycelia of *M. alpina* 1S-4, and about 80% of that was consumed by the fungus. Twelve percent of the consumed *n*-pentadecane was converted and accumulated as mycelial fatty acids, but the rest of the consumed *n*-pentadecane is considered to have also been converted to pentadecanoic acid, which is then metabolized to various cellular components. This seems to be

reflected by the fact that the percentage of C_{16:0} acid in the TG-fraction derived from the mycelia grown in the NA medium was very high (56.8%) (Table 5). The PUFA contents then were low (C_{18:4}, 1.7%; and C_{20:4}, 0.4%). On the other hand, the PL-fraction of the same mycelia contained relatively lower percentages of C_{18:0} (15.4%) and C_{17:0} (7.5%) acids and higher percentage of C_{19:4} (14.0%) and C_{20:4} (3.9%). Since all odd chain fatty acids were found not only in the TG-fraction but also in the PL-fraction, they are considered to occur and function as constituents in biomembranes. The higher percentages of PUFAs such as C_{19:4} and C_{20:4} acids in the PL-fraction show that these PUFAs are necessary for the functions of a biomembrane and are probably selectively incorporated into the biomembrane.

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